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Abstract

Background: Allergic asthma is a chronic inflammatory disease that is characterized by airway hyper responsiveness (AHR), infiltration of Th2 cells in lungs and high levels of circulating IgE. Allergen-specific immunotherapy (SIT), in which patients are rendered tolerant by exposure to steadily increasing doses of the allergen, is the only curative treatment to date. Unfortunately, SIT is not suitable for treating multi-sensitized patients, and some allergens are too immunogenic to be used in desensitization protocols.

Objective: To investigate whether, and to understand how, regulatory $CD4^+$ T cells (T_{reg}) specific for a third-party "drug" antigen could control allergic immune responses and lung inflammation.

Methods: Mice were tolerized to ovalbumin (OVA), sensitized to ragweed, and eventually challenged with aerosols of ragweed alone or ragweed and OVA together. Animals were then monitored for cardinal features of allergic asthma including AHR and infiltration of Th2 cells in lungs. In additional experiments aimed at elucidating the mechanisms of OVA-induced suppression, OVA-tolerized mice were sensitized with the LACK model antigen, challenged with LACK alone or LACK and OVA together, and LACK-specific T cells were visualized by flow cytometry.

Results: In both the ragweed and the LACK model, allergen-induced airway inflammation and AHR were strongly reduced in mice challenged with both the allergen and OVA compared to mice challenged with the allergen alone. OVA-induced protection did not result from competition between OVA and the allergen, was mediated by OVA-specific $CD25^+$ T_{reg}, required both CTLA-4 and ICOS signaling, and was partially dependent on IL-10. Bystander suppression was associated with reduced proliferation of allergen-specific Th2 cells and decreased numbers of airway DC migrating to the lungs.

Conclusion: Our results demonstrate that T_{reg} specific for a third-party drug antigen could control allergic immune responses and lung inflammation when re-stimulated *in vivo.*

Clinical implications: This study paves the way for the development of a novel therapeutic strategy that could control allergen-specific Th2 responses in patients with allergic asthma.

Capsule summary: This study provides the proof of concept that bystander suppression mediated by T_{reg} specific for a third-party drug antigen could be used as an efficient strategy to control allergen-specific Th2 cells and asthma symptoms in allergic individuals.

Key words: asthma, immune tolerance, airway inflammation, Th2, T_{reg}, specific immunotherapy

Abbreviations: **Ag**, antigen; **APC**, antigen-presenting cells; **AHR**, airway hyperresponsiveness; **Alum**, aluminium hydroxide; **BAL**, bronchoalveolar lavages; **BALF**, bronchoalveolar lavage fluids; **BSA,** bovine serum albumin**; CFSE**, carboxyfluorescence diacetate succinimidly ester; **DCs**, dendritic cells; **ELISA**, enzyme-Linked immunosorbent assay; **FACS**, fluorescence activated cell sorter; **FITC**, fluoresceinisothiocyanate; **LACK**, *Leishmania* homolog of receptors for activated c kinase antigen; **LN**, lymph nodes; **Ig**, immunoglobulin; **IFN**, interferon; **IL**, interleukin ; **i.p.**, intraperitoneal ; **i.n.**, intranasal; **MCH**, methacholine; **MedLN**, mediastinal LN; **OVA**, ovalbumin; **PCR**, polymerase chain reaction; **PE**, phycoerythrin; **PBS**, phosphate-buffered saline; **PLN**, popliteal LN; **RNA**, ribonucleic acid; **SIT**, specific immunotherapy; $\mathbf{T_{eff}}$, effector T cells; **Tg**, transgenic; **Th**, T helper ; $\mathbf{T_{reg}}$, regulatory T cells; **WT**, wild type

Introduction

Allergic diseases affect up to 30% of the population and their prevalence has steadily increased in recent decades probably due to numerous changes in the environment. Among allergic diseases, asthma is a chronic inflammation of the lungs caused by an inappropriate immune response to a single or multiple airborne allergens. This pathology has a substantial economic burden for which the only curative and specific method of treatment to this day is allergen-specific immunotherapy (SIT). SIT involves the administration by either subcutaneous injection or mucosal application of increasing doses of the allergen to which the patient is allergic to. Unfortunately, SIT is unsuitable for treating multi-sensitized patients, and some allergens are too immunogenic to be used in desensitization protocols.

SIT induces a state of peripheral tolerance characterized mainly by the generation of allergen-specific T_{reg}, suppressed effector cell proliferation and cytokine production against major allergens [1, 2]. Indeed, IL-10 and/or TGF-β producing T_{reg} are the key factors for specific immunotherapy in humans, considered as a model of tolerance induction [3-5]. It has been shown that treating naive (non-sensitized) mice with OVA aerosols leads to IgE-unresponsiveness to OVA [6], and induces the development of OVA-specific T_{reg} which prevented the development of asthma upon subsequent sensitization and challenge with OVA [7]. Moreover, lung draining lymph node (LN) dendritic cells (DC) first encountering an inhaled antigen transiently produced IL-10 [7]. These phenotypically mature DC induce the development of $CD4^+$ T_{reg} that also produce high amounts of IL-10 [8]. TGF-β-expressing T_{reg} also play a role for tolerance induction to inhaled antigens [5, 9]. Interestingly, it has been suggested that tolerance induced by SIT was not only limited to the administered allergen but also conferred protection against other allergens [10, 11]. This non-specific and beneficial action of SIT could be explained by the activation of bystander T_{reg}.

Bystander immunosuppression was first described by Bullock *et al.* as a process in which antigen (Ag)-specific T_{reg} inhibit T effector cell responses directed to both the targeted Ag and to a co-localized third-party Ag [12]. Due to the requirement that the tolerogen and the Ag have to be physically linked, i.e. presented by the same antigen-presenting cell (APCs), in order for suppression to occur, the terms « linked suppression » or « linked recognition » were coined. In the field of oral tolerance, the term « bystander suppression » was introduced to describe an inhibition of a T cell memory response as a result of a regulatory response to an unrelated but

colocalized tolerogen [13, 14]. In bystander suppression, the tolerogen and third-party Ag do not need to be presented by the same APC. In this case, soluble mediators induce suppression of the response directed to the third-party Ag [13, 15-17].

In the present study, we have sought to investigate whether T_{reg} specific for a third-party "drug" antigen could control allergic immune responses and lung inflammation in mice. To this aim, mice were tolerized to OVA, sensitized to ragweed, and eventually challenged with aerosols of ragweed alone or ragweed and OVA together.

Results

Exposure to OVA aerosols inhibits ragweed- and LACK-induced allergic asthma in OVA-tolerized mice

To determine whether T_{reg} specific for a third-party antigen could control allergic airway inflammation in mice, mice were tolerized to OVA through intranasal administrations, sensitized to ragweed and further exposed to aerosols of ragweed alone or ragweed and OVA together (**Fig 1, *A***). When compared to the group challenged with ragweed only, mice exposed to both ragweed and OVA showed decreased AHR (**Fig 1, *B***) and reduced numbers of total cells, eosinophils and lymphocytes in broncho alveolar lavage fluids (BALF) (**Fig 1, *C***). We further analyzed airway-infiltrating T cells for expression of the Th2- marker, T1/ST2 (IL-33Ra). Compared to the mice challenged with ragweed alone, the number of $T1/ST2^+$ $CD4^+$ T cells was reduced in the BALF of mice challenged with ragweed and OVA aerosols (**Fig 1, *D***). The amounts of IL-5 and IL-13 in the lungs were also reduced upon challenge with ragweed and OVA while IFN-γ levels remained low and similar in both groups (**Fig 1, *E***). This phenomenon was not observed in mice than have not been tolerized to OVA prior to ragweed sensitization (Supplementary Figure 1) further supporting a role for OVA-specific T_{reg} in this phenomenon and ruling out the possibility that it could result from competition between OVA and ragweed for antigen presentation.

To generalize our findings and to further dissect the mechanisms involved, we switched to another experimental model in which BALB/c mice were sensitized to the model antigen LACK, tolerized to OVA through intranasal administration, and further exposed to aerosols of LACK alone, or LACK and OVA (**Fig 2, *A***). As observed with ragweed, mice exposed to both LACK/OVA showed decreased AHR (**Fig 2, *B***), and reduced total numbers of cells, eosinophils and lymphocytes in BALF (**Fig 2, *C***). Compared to control mice challenged with LACK alone, both the frequency and the number of $T1/ST2^+$ $CD4^+$ T cells were reduced in the BALF of mice challenged with LACK/OVA aerosols (**Fig 2, *D***). The amounts of IL-4, and IL-13 in the BALF were also reduced upon challenge with LACK/OVA aerosols while IFN-γ levels remained low and similar in both groups (**Fig 2, *E***). The lungs of LACK/OVA-challenged mice contained less IL-4-, and IL-5- secreting $CD4^+$ T cells than those challenged with LACK only as demonstrated by intracellular cytokine staining of lung cells upon LACK restimulation (**Fig 2, *F***). In contrast, the amounts of LACK-specific -IgE and -IgG1 were not affected by additional OVA exposure

(**Fig 2, *G***). Altogether, our results suggested that exposure to OVA aerosols could inhibit allergic airway inflammation and allergen-specific Th2 immune responses in mice that have been tolerized with OVA prior to sensitization and challenge with either ragweed or LACK.

The inhibition of LACK-induced airway inflammation in OVA-tolerized mice is mediated by OVA-specific $CD25^+$ T_{reg}

Previous experiments have shown that the intranasal administration of OVA to BALB/c mice induces OVA-specific T_{reg} that express CD25. To investigate whether these cells were responsible for the inhibition of LACK-induced airway inflammation in OVA-tolerized LACK-sensitized mice, $CD4^+$ T cells were purified from the spleen of mice that have been previously tolerized to OVA or bovine serum albumine (BSA), or treated with PBS. Cells were injected into LACK-sensitized recipients that were further challenged with both LACK and OVA **(Fig 3, *A*)**. Compared to mice injected with $CD4^+$ T cells purified from BSA-tolerized or PBS-treated donors, mice injected with $CD4^+$ T cells from OVA-tolerized mice exhibited decreased AHR **(Fig 3, *B*)**, reduced numbers of total cells, eosinophils and lymphocytes **(Fig 3, *C*)** and $T1/ST2^+$ $CD4^+$ T cells in BALF **(Fig 3, *D*)**, and reduced numbers of IL-4 and IL-5-secreting $CD4^+$ T cells in lungs **(Fig 3, *E*)**. In striking contrast, no inhibition of LACK-induced airway inflammation or LACK-specific Th2 immune responses were observed when $CD4^+$ T cells from OVA-tolerized mice depleted of $CD25^+$ cells prior to injection into LACK-sensitized mice. Lastly, both airway inflammation and LACK-specific Th2 immune responses were inhibited when recipient mice were injected with $CD25^+$ $CD4^+$ T cells purified from the spleen of OVA-tolerized mice **(Fig 3, *F-I*).** Therefore, the inhibition of LACK-induced airway inflammation that was observed in OVA-tolerized mice upon challenge with OVA relied on the activation of OVA-specific $CD25^+$ T_{reg}.

CTLA-4, ICOS, and IL-10 dependency of OVA-specific T_{reg} in the control of LACK-induced asthma

T_{reg} mediate suppression through various mechanisms including the secretion of inhibitory cytokines such as IL-10, the induction of cytolysis, metabolic disruption and the inhibition of antigen presentation by dendritic cells (DCs) through a CTLA-4-dependent mechanism [18].

Furthermore, it was recently shown that ICOS mediated the generation and function of $CD25^+$ $CD4^+$ $FoxP3^+$ T_{reg} conveying respiratory tolerance [19], and that ICOS expression defined a subset of IL-10 secreting T_{reg} [20] and was required for the production of IL-10 by these cells [21]. Therefore, we sought to elucidate the role of CTLA-4, ICOS and IL-10 in the inhibition of LACK-induced airway inflammation induced by OVA aerosols. To this aim, LACK-sensitized OVA-tolerized mice were challenged with both LACK and OVA and treated or not with blocking mAbs directed to CTLA-4, ICOS or IL-10R (**Fig 4, *A***). CTLA-4 blockade prevented the inhibition of AHR induced by OVA aerosols, as well as the reduction in the number of total cells, eosinophils, lymphocytes and $T1/ST2^+$ Th2 cells in BALF, and IL-4- and IL-5-producing LACK-specific $CD4^+$ in lungs (**Fig 4, *B*-*D***). Likewise, blocking ICOS/ICOSL interactions abolished the protection that was induced by OVA aerosols in OVA-tolerized LACK-sensitized mice but did not have any detectable effect in mice that have not been tolerized to OVA (**Fig 4, *F*, *G***). In contrast to anti-CTLA-4 and anti-ICOS mAbs that restored both AHR and allergic airway inflammation in OVA-tolerized mice exposed to OVA, anti-IL-10R mAbs restored AHR but not allergic airway inflammation (**Fig 4, *F*, *G***). Therefore, the inhibition of AHR and airway inflammation that was induced by OVA aerosols in OVA-tolerized mice upon sensitization and challenge with LACK was dependent on both CTLA-4, ICOS and partially on IL-10.

OVA-specific T_{reg} inhibited the proliferation of LACK-specific Th2 cells and the subsequent airway inflammation

To further dissect the molecular mechanisms by which OVA-specific T_{reg} inhibited LACK-induced allergic asthma, $CD4^+$ T cells from $Thy1.1^+$ LACK-specific WT15 TCR transgenic mice were differentiated *in vitro* under Th2 polarizing conditions, labeled with CFSE, and injected into OVA- or BSA- tolerized recipients before being challenged with LACK/OVA (**Fig 5, *A***). As compared to BSA-tolerized mice, OVA-tolerized animals injected with LACK-specific Th2 cells exhibited decreased AHR (**Fig 5, *B***), and reduced numbers of eosinophils and lymphocytes in the BALF (**Fig 5, *C***). In addition, both the frequency and the number of donor Th2 cells were reduced in the BALF, lung and MLN, but not in the blood of OVA-tolerized mice compared to BSA-tolerized mice (**Fig 5, *D***). Furthermore, LACK-specific Th2 cells had undergone more divisions in BSA-tolerized mice than in OVA-tolerized mice (**Fig 5, *E***) further suggesting that OVA aerosols inhibited the proliferation of LACK-specific Th2 cells possibly at the level of

antigen presentation. To directly test this latter hypothesis, LACK-specific Th2 cells were injected into either OVA- or BSA-tolerized mice, and further challenged with both LACK and OVA. Cells were then purified from the mediastinal LN (MedLN) and distal popliteal LN (PLN) and incubated with CFSE-labeled LACK-specific WT15 $CD4^+$ T cells to assess their ability to induce proliferation. As expected, PLN cells did not induce T cell proliferation (**Fig 5, *F***).. Furthermore, LACK-specific T cells proliferated more vigorously when incubated with MedLN cells from BSA-tolerized mice than when incubated with MedLN cells from OVA-tolerized mice (**Fig 5, *F***). To investigate whether this phenomenon resulted from quantitative (i.e. different number of LACK-loaded DCs in OVA-tolerized and BSA-tolerized mice) or qualitative differences (i.e. similar number of LACK-loaded DCs in OVA-tolerized and BSA-tolerized mice but different ability to induce T cell proliferation), we administered FITC-labeled latex beads to OVA- and BSA-tolerized mice at the time of the challenge with LACK and OVA, and we measure the frequency of $bead^+$ DCs in MedLN 16 hrs later. While $bead^+$ DCs were readily detected in the MedLN of both OVA- and BSA-tolerized mice and expressed similar surface levels of CD80, CD86, OX40L and MHC class II molecules (not shown), the frequency of $bead^+$ DCs was decreased from 4.7 ± 0.7 % in BSA-tolerized mice to 1.4 ± 0.3 % in OVA-tolerized animals (**Fig 5, *H***). Altogether, our data suggested that OVA-specific T_{reg} inhibited the proliferation of LACK-specific Th2 cells by preventing the migration of airway DCs to MedLN.

Discussion

In his paper, we have demonstrated that OVA-specific T_{reg} could inhibit allergic airway inflammation induced by sensitization and challenge with ragweed. OVA-specific T_{reg} were also efficient to protect mice from allergic airway inflammation induced by the immunodominant LACK antigen further validating our finding to second allergen. In addition, BSA-tolerized mice were protected from LACK-induced allergic asthma when challenged with both LACK and BSA further suggesting that the phenomenon that we have observed was not restricted to OVA-specific T_{reg} (data not shown). Interestingly, the phenomenon that we have described in this paper may explain the protective effects of SIT against unrelated allergens other than the one primarily targeted as reported in several epidemiological studies [10, 11, 22].

In the LACK model, we have found that the number of allergen-specific Th2 cells in BALF, the frequency and number of IL-4 and IL-5 producing $CD4^+$ T cells as well as the IL-4, IL-5, and IL-13 amounts in lungs were decreased in mice challenged with both LACK and OVA compared to mice challenged with LACK alone. OVA-induced protection did not result from a competition between OVA and LACK for antigen presentation because it was only observed in mice that have been previously tolerized with OVA. In contrast, experiments in which $CD4^+$ T cells were purified from the spleen of PBS-treated, BSA- or OVA-tolerized mice and injected into LACK-sensitized recipient mice showed that protection was antigen-specific and mediated by T_{reg}.

Further experiments showed that CTLA-4 was absolutely required for OVA-induced suppression of LACK-mediated asthma. While we did not elucidate the mechanisms by which CTLA-4 acted, CTLA-4 was shown to be essential for the function of naturally occurring T_{reg} which constitutively express this molecule. Indeed, CTLA-4 deficiency in T_{reg} impaired both their *in vivo* and *in vitro* suppressive function. In addition, CTLA-4 prevented CD28 signaling in effector T cells (T_{eff}) by competing with CD80 and CD86, and/or induced the synthesis of the tryptophan catabolizing enzyme indoleamine 2,3-dioxygenase (IDO) by the APCs leading to T-cell suppression by both local depletion of tryptophan and induction of apoptosis via tryptophan catabolites [23]. While we were unable to detect increased death of LACK-specific T cells in OVA-tolerized mice challenged with both OVA and LACK (not shown), we cannot rule out the possibility that this phenomenon was partially responsible for OVA-induced protection.

Blocking ICOS-ICOSL interactions resulted in the same effect as CTLA-4 inhibition, a result in agreement with a previous study showing that $ICOS^{-/-}$ T_{reg} did not confer protection upon transfer to asthmatic mice demonstrating a crucial role of ICOS in their suppressive function [19]. As ICOS was shown to define a subset of IL-10-producing T_{reg}, we next investigated whether OVA-induced suppression of the disease required IL-10. IL-10R blockade prevented OVA-induced suppression of AHR, but did not affect LACK-induced inflammation in our model. It remains to be determined whether IL-10 is produced by the $CD25^+$ T_{reg} or by other cells as previously described Kearley *et al.* [24]. Taken together, these data underline the multiple and complex effects of $CD25^+$ T_{reg}. While these cells have been proposed to act via cell contact-dependent mechanisms *in vitro,* these cells have been proposed to work through various mechanisms including inhibitory cytokines and non-cytokine-dependent mechanisms *in vivo*, depending on the experimental conditions [25]. Our findings also suggest that inflammation and AHR can be uncoupled and are in agreement with previous studies that demonstrate that effects on inflammation are not always predictive of AHR changes [26-29]. Indeed, this might also hold true in human asthma, in which anti-IL-5 mAb treatment reduced blood and lung eosinophilia but did not affect lung function [30].

Adoptive transfer of CFSE-labeled LACK-specific Th2 cells into OVA-tolerized recipient mice demonstrated that OVA-specific T_{reg} significantly reduced the number of LACK-specific Th2 cells in the BALF, lung and MLN by affecting their proliferation. Imaging data in mice have showed that T_{reg} do not directly interact with T_{eff} but rather with DC, altering the latter and diminishing subsequent DC-T_{eff} cells conjugate formation *in vivo* [31, 32] a phenomenon that could explain our results. In addition, Derks and colleagues have envisioned two hypotheses of APC function in bystander suppression [33]: a passive APC model, in which the APCs would present MHC-peptide to the T_{reg}, stimulating them to produce immunosuppressive cytokines that would further binds their cognate receptors on the third-party T_{eff}, or an active APC model, in which the APCs would propagate regulatory effects from the T_{reg} to the T_{eff} through various APC products. These two hypotheses remain to be tested in our model.

Our results demonstrate that T_{reg} specific for a third-party drug antigen could control allergic immune responses and lung inflammation when re-stimulated *in vivo.* This study paves the way for the development of a novel therapeutic strategy that could control allergen-specific

Th2 responses in patients with allergic asthma, and more specifically in patients who are sensitized to multiple allergens.

FIGURE LEGENDS

Figure 1. AHR, airway inflammation and cytokine levels in ragweed-sensitized, OVA-tolerized mice upon ragweed challenge. (**A**) Experimental protocol. Mice were treated with three i.n. injections of OVA, and four i.n. injections of ragweed. Mice were then either challenged with ragweed or ragweed/OVA, and analyzed one and two days after the last i.n. injection. (**B**) AHR. Whole body plethysmography in mice exposed to ragweed (filled squares), ragweed/OVA (empty circles), or PBS (crosses). (**C**) Number and phenotype of BALF cells. BALF cells were analyzed by FACS in mice exposed to ragweed only (black bars), to both ragweed and OVA aerosols (empty bars) or to PBS (grey bars). Eosinophils, E; neutrophils, N; lymphocytes, L; macrophages, M. (**D**) Frequency and number of Th2 cells in the airways. BALF cells were stained with CD4 and T1/ST2 mAbs and analyzed by FACS. (**E**) Cytokine levels in lung cells. IL-4, IL-5 and IFN-γ levels were assessed by ELISA after *in vitro* stimulation with LACK protein (0.1mg/ml), anti-CD28 (1μg/ml) and brefeldin A (5μg/ml). Data are expressed as mean ± s.e.m. of 2 experiments with n=8 mice per group. n.s., non significant; * $P<0.05$; ** $P<0.01$.

Figure 2. AHR, airway inflammation and cytokine levels in OVA-tolerized mice exposed to both OVA and LACK aerosols. (**A**) Experimental protocol. Mice were sensitized with two intra-peritoneal injections of LACK in Alum, treated with three i.n. injections of OVA, and challenged daily for 5 days with LACK aerosols or LACK/OVA aerosols. Mice were analyzed one and two days after the last aerosol. (**B**) AHR. Whole body plethysmography (right), and dynamic lung resistance and compliance (left) were monitored in mice exposed to LACK aerosols (filled squares), LACK/OVA aerosols (empty circles) or PBS (crosses, dashed line). (**C**) Number and phenotype of BALF cells. BALF cells were counted and analyzed by FACS in mice exposed to PBS (dashed bars), LACK (black bars) or LACK/OVA aerosols (empty bars). Data show the number of eosinophils (E), neutrophils (N), lymphocytes (L), macrophages (M). (**D**) Frequency and number of Th2 cells in the airways. BALF cells were stained with anti-CD4, CD3 and T1/ST2 mAbs and analyzed by FACS. Data show representative FACS profiles, numbers indicate the mean frequency ± s.e.m and histograms show the absolute numbers of $T1ST2^+$ $CD4^+$ T cells for the indicated groups. (**E**) Cytokine levels in BALF. Mice were analyzed for IL-4, IL-5, IL-13 and IFN-γ by cytometric bead array (CBA). (**F**) Cytokine secretion by lung $CD4^+$ T cells.

IL-4, and IL-5-secreting $CD4^+$ T cells were assessed by FACS after *in vitro* stimulation with LACK protein (0.1mg/ml), anti-CD28 (1μg/ml) and brefeldin A (5μg/ml). Data show representative FACS profiles, numbers indicate the mean frequency ± s.e.m and histograms show the absolute numbers for the indicated groups. (**G**) Immunoglobulins. Levels of serum LACK-specific -IgE and -IgG1 were assessed in mice upon challenge with LACK or LACK/OVA aerosols. All data show either individual mice with bar indicating the mean, with n = 6 mice per group pooled from four different experiments. n.s., non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.0001$.

Figure 3. AHR, airway inflammation and cytokine levels in mice injected with $CD4^+$ T cells from OVA-tolerized mice. (**A**) Experimental protocol. Mice were sensitized with two i.p. injections of LACK in Alum, and injected 9 d later with 4 X 10^6 $CD4^+$ T cells (**A-E**), or 4 X 10^6 $CD25^-CD4^+$ T cells (**F-I**) or 1.5 X 10^6 $CD25^+CD4^+$ T cells (**F-I**) prepared from the spleen of mice exposed to OVA, BSA, or PBS. Sensitized mice were then challenged with LACK/OVA aerosols for five days and analyzed one and two days after the last aerosol. (**B, F**) AHR. Whole body plethysmography was monitored in the indicated mice challenged to LACK/OVA aerosols in response to increased doses of inhaled methacholine. Control mice (vehicle) were sensitized with LACK, non-transferred and challenged with PBS. (**C, G**) Number and phenotype of BALF cells. BALF cells were analyzed by FACS for the number of eosinophils (E), neutrophils (N), lymphocytes (L), macrophages (M). (**D, H**) Frequency and number of Th2 cells in the airways. BALF cells were stained with anti-CD3, -CD4 and T1/ST2 mAbs and analyzed by FACS. (**E, I**) Cytokine secretion by lung $CD4^+$ T cells. IL-4, and IL-5-secreting $CD4^+$ T cells were assessed by FACS after *in vitro* stimulation with LACK protein (0.1mg/ml), anti-CD28 (1μg/ml) and brefeldin A (5μg/ml). Data show numbers of IL-4 and IL-5-secreting $CD4^+$ T cells in lungs. All data show either individual mice or are expressed as mean ± s.e.m, with n = 5 mice per group pooled from two experiments. P values have been calculated by comparing OVA-tolerized mice to BSA-tolerized mice, ns, non significant, * $P<0.05$, ** $P<0.01$, *** $P<0.0001$.

Figure 4: **AHR and airway inflammation in mice treated with anti-CTLA-4, anti-IL-10R, or anti-ICOS.** (**A**) Experimental protocol. Mice were sensitized with two i.p. injections of LACK in

Alum, treated with three i.n. injections of OVA, treated or not with anti- CTLA-4 mAb, -IL-10R, -ICOS, or IgG1 isotype mAb at the indicated time, and challenged daily for 5 days with LACK or LACK/OVA aerosols. Mice were analyzed one and two days after the last aerosol. (**B**) and (**F**) AHR. Whole body plethysmography in response to increasing doses of inhaled methacholine in the indicated groups of mice. (**C**) and (**G**) Number and phenotype of BALF cells. BALF cells were analyzed by FACS in the indicated groups of mice. Eosinophils, E; neutrophils, N; lymphocytes, L; macrophages, M. (**D**). Number of Th2 cells in the airways. BALF cells were stained with anti -CD3, -CD4 and T1/ST2 mAbs and analyzed by FACS. (**E**). IL-4, and IL-5-secreting $CD4^+$ T cells were assessed by FACS after *in vitro* stimulation with LACK protein (0.1mg/ml), anti-CD28 (1μg/ml) and brefeldin A (5μg/ml). Data are expressed as mean ± s.e.m of 3 experiments with n=6 mice per group. n.s., non significant; * $P<0.05$; ** $P<0.01$; *** $P<0.0001$.

Figure 5. AHR, airway inflammation, cytokine levels and T cell proliferation in OVA-tolerized mice injected with LACK-specific Th2 cells and exposed to LACK/OVA. (**A**) Experimental protocol. Mice were treated with three i.n. injections of OVA or BSA, injected with 1.5 X 10^6 CFSE-labeled $Thy1.1^+$ LACK-specific Th2 cells. Mice were injected intranasally 2 d later with LACK/OVA and analyzed 4 and 5 days later. (**B**) AHR. Whole body plethysmography was monitored in mice tolerized to OVA (empty circles) or BSA (filled squares, and crosses) and exposed to aerosols of LACK/OVA (full lines) or to PBS (dashed lines). Data are expressed as mean ± s.e.m. (**C**) Number and phenotype of BALF cells. BALF cells were analyzed by FACS in the indicated groups of mice. Eosinophils, E; neutrophils, N; lymphocytes, L; macrophages, M. Data are expressed as mean ± SEM of 3 experiments with n=6-8 mice per group. (**D**) Numbers of $Thy1.1^+$ LACK-specific Th2 cells in BALF, lung, and medLN and frequency in the blood of BSA- (filled bars) or OVA- (empty bars) tolerized mice challenged with LACK/OVA aerosols. (**E**) Representative plots of CFSE (left panels) and MFI of CFSE (right panels) of the indicated mice. (**F**) *In vitro* antigen presentation assay. Whole cell suspensions prepared from the MedLN and PLN of OVA- or BSA-exposed mice were incubated for 3 days with CFSE-labeled LACK-specific Th2 cells. Data show representative CFSE plots for the indicated mice with the frequency of divided cells as mean ± s.e.m of 2 experiments with n=6 mice per group. (**G**) Mice underwent the same protocol as shown in panel A, but also received fluorescent latex beads

during LACK/OVA challenge. Beads^{+}CD11c^{+} migratory DCs were analyzed by FACS in the MedLN of BSA- (filled bars) or OVA- (empty bars) tolerized mice challenged with LACK/OVA aerosols. n.s., non significant; * P<0.05; ** P<0.01; *** P<0.0001.

Supplementary Figure 1. AHR and airway inflammation in LACK-sensitized mice challenged with LACK alone or LACK and OVA aerosols. (**A**) Experimental protocol. Mice were sensitized with two i.p. injections of LACK in Alum, and challenged daily for 5 days with LACK or LACK/OVA aerosols, or PBS. Mice were analyzed one and two days after the last aerosol. (**B**) AHR. Whole body plethysmography in mice exposed to aerosols of LACK (filled squares), LACK/OVA (empty circles) or PBS (crosses). (**C**) Number and phenotype of BALF cells. BALF cells were analyzed by FACS in mice exposed to LACK only (black bars), to both LACK and OVA aerosols (empty bars) or to PBS (grey bars). Eosinophils, E; neutrophils, N; lymphocytes, L; macrophages, M. Data are expressed as mean ± s.e.m. of 2 experiments with n=8 mice per group. n.s., non significant.

METHODS

Mice. 6-week old BALB/c mice were purchased from The Centre d'Elevage Janvier (France) and housed under SPF conditions. LACK TCR transgenic mice (WT15 RAG-1 KO) on the BALB/c background as previously described [34] were bred in our animal facility at the Institut de Pharmacologie Moleculaire et Cellulaire (Valbonne, France). In this study, WT15 transgenic mice were further crossed onto RAG-$1^{-/-}$ Thy$1.1^{+/+}$ BALB/c mice. All experimental protocols were approved by the local animal ethic committee.

Reagents. LACK recombinant protein was produced in *E. coli*, purified as described [35], and detoxified using an Endotrap column (Profos). Lipopolysaccharide contents in LACK protein were below 5 ng/mg as determined using Limulus Amoebocyte Lysate (LAL) assay (Pierce). $LACK_{156-173}$ peptide was purchased from Mimotopes. T1/ST2 mAbs were purchased from MD Biosciences. Monoclonal antibodies to CD3, CD4, CD25, Thy1.1, CD11c, IA/IE, CD80, CD86, IL-4 and IL-5 were purchased from BD Biosciences.

Induction of allergic asthma and tolerization to OVA. Sensitization was performed by 2 intraperitojneal (i.p.) injections of 10 µg of LACK in 2 mg of Aluminium hydroxide (Alum) (Pierce) at day 0 and 7. On days 12, 13 and 14, mice were tolerized to OVA by injecting i.n. 100 µg of LPS-free OVA (Profos) as described [7]. From day 23 to day 27, mice were either exposed to LACK (0.15%) or to LACK plus OVA (0.2%) aerosols (administered 8 hour apart) for 20 min using an ultrasonic nebulizer (Ultramed, Medicalia). Mice were analyzed on day 28 and 29 for AHR and airway inflammation, respectively. When indicated, mice were injected with either 0.5 mg of anti-CTLA-4 mAb (9H10), anti-ICOS (17G9), or anti-IL-10R (1B1.3A) every other day over the challenge period starting one day before the first aerosol. For ragweed-induced asthma, mice were first tolerized to OVA by receiving i.n. injections of OVA on days 0, 1, and 2 and further sensitized to ragweed via i.n. administrations of 25 µg ragweed (Greer laboratories) on days 11, 15, 19, and 23. Mice received a last challenge of ragweed on day 27 or ragweed and OVA on days 26 and 27. Mice were analyzed on day 28 for AHR and on day 29 for airway inflammation.

Th2 cell transfers. In some experiments, mice were first tolerized to OVA, BSA or PBS and injected i.v on day 11 with LACK-specific $CD4^+$ Th2 cells. Mice were challenged 24 hours later with a single i.n. injection of LACK (30 μg) and OVA (100 μg), and assessed for AHR, and immunological parameters 3 and 4 days later, respectively.

AHR. For non-invasive measurements, mice were analyzed one day after the last aerosol challenge using whole body plethysmography as described [36]. Invasive measurements of dynamic lung resistance and compliance were performed one day after the last aerosol challenge using a Flexivent apparatus (SCIREQ, Emka Technologies) as previously described [37]. Briefly, mice were anesthetized (5 ml/kg Dormitor 10 % (Medetomidine, Pfizer) - Imalgene 10% (Ketamine, Merial) tracheotomized, paralyzed (5 ml/kg Pavulon 1% (Pancuronium bromide, Organon) and immediately intubated with an 18-G catheter, followed by mechanical ventilation. Respiratory frequency was set at 150 breaths/min with a tidal volume of 0.2 ml, and a positive-end expiratory pressure of 2 ml H_2O was applied. Increasing concentrations of methacholine (0-24 mg/ml) were administered at the rate of 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 10 ms, via a nebulizer aerosol system with a 2.5-4 μm aerosol particle size generated by a nebulizer head (Aeroneb, Aerogen). Baseline resistance was restored before administering the subsequent doses of methacholine.

Analysis of BALF cells. Mice were bled and a canula was inserted into the trachea. Lungs were washed 3 times with 1 ml of warmed PBS. For differential BALF cell counts, cells were stained with mAb anti-CCR3 (R&D), anti-Gr1, anti-CD3 and anti-CD19 mAbs (Becton Dickinson, BD) and analyzed by FACS using a FACScalibur flow cytometer and Cellquest software. Eosinophils were defined as $CCR3^+$ $CD3^-CD19^-$, neutrophils as $Gr\text{-}1^{high}$ $CD3^-CD19^-$, lymphocytes as $CD3^+CD19^+$ and alveolar macrophages as large autofluorescent cells.

Serum antibody measurements. Serum LACK-specific IgG1 and IgE were measured by ELISA. For IgG1 quantification, antigen-coated Maxisorp plates (Nunc) were incubated with serial dilution of sera and biotinylated anti-IgG1 mAb (BD). For antigen-specific IgE, plates were first coated with the respective capture mAb (BD), and incubated with serum dilutions. Biotinylated-LACK antigen was then added. HRP-conjugated streptavidin (BD) and TMB (KPL) were used for detection.

Tissue processing. Lungs, LN or spleens were cut to small pieces in HBSS containing 400 U type I collagenase and 1 mg/ml DNAse I and digested for 30 min at 37°C. Cells were strained through a 70 μm cell strainer. Erythrocytes were lysed with ACK lysis buffer.

Cytokine assays. Lung samples were homogenized in C^{2+} and Mg^{2+} free HBSS. BAL and lung supernatants were used. Multiplex IL-4, IL-5, IL-13 and IFN-γ analysis were performed with CBA using FACS array (BD Bisociences). For intracellular staining, cells were incubated with 100 μg/ml LACK and 1 μg/ml of anti-CD28 (BD) for 6 h. Brefeldin A (5 μg/ml, Sigma) was added during the last 4 h. Cells were then stained with anti-CD4 mAb, fixed, permeabilized using cytofix/cytoperm reagent (BD), stained with anti-IL-4, or IL-5 (BD) and analyzed by FACS.

$CD4^+$ T cell transfer. Donor mice were tolerized to OVA, BSA or PBS as described above. Cells were prepared from spleens 21 days later, and $CD4^+$ T cells were enriched by negative depletion using CD4 isolation kit (Dynal) and further sorted using a high-speed sorter VANTAGE $SETLO^+$ flow cytometer (BD) after staining with anti-CD3 and anti-CD4 mAbs. CD4 purity was > 95%. In some experiments, enriched $CD4^+$ T cells were stained with antibodies to CD25, CD4 and CD3, and $CD25^-$ and $CD25^+$ $CD4^+$ T cell populations were sorted by FACS. Sorted cells were then injected i.v. into sensitized mice (4×10^6 or 1.5×10^6 cells per mouse, respectively).

Statistic analysis. ANOVA for repeated measures was used to determine the levels of difference between groups of mice for plethysmography measurements. Comparisons for all pairs were performed by Mann-Whitney U test. Significance levels were set at a *P* value of 0.05.

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COMPETING INTEREST STATEMENT

The authors declare no competing financial interests.

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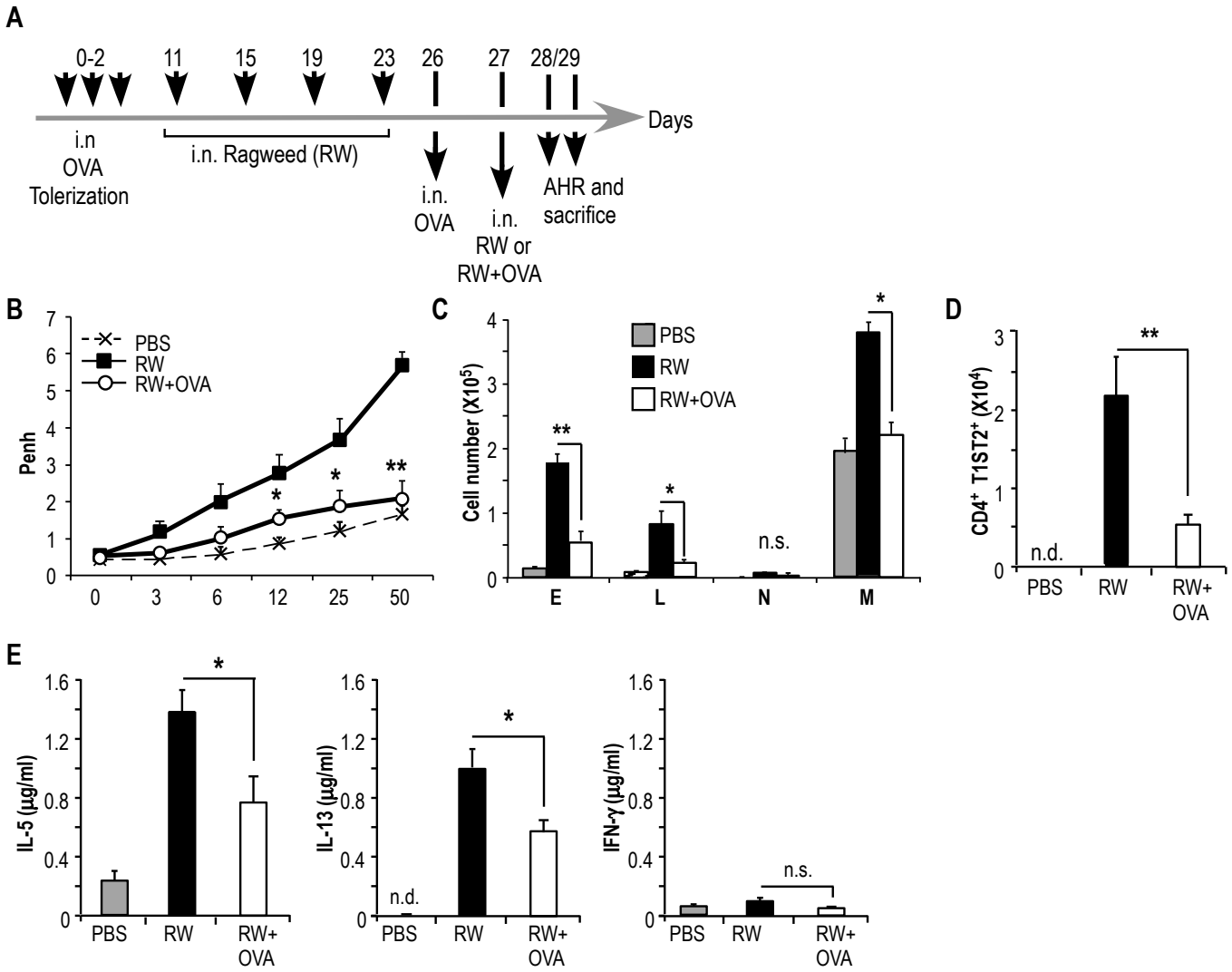

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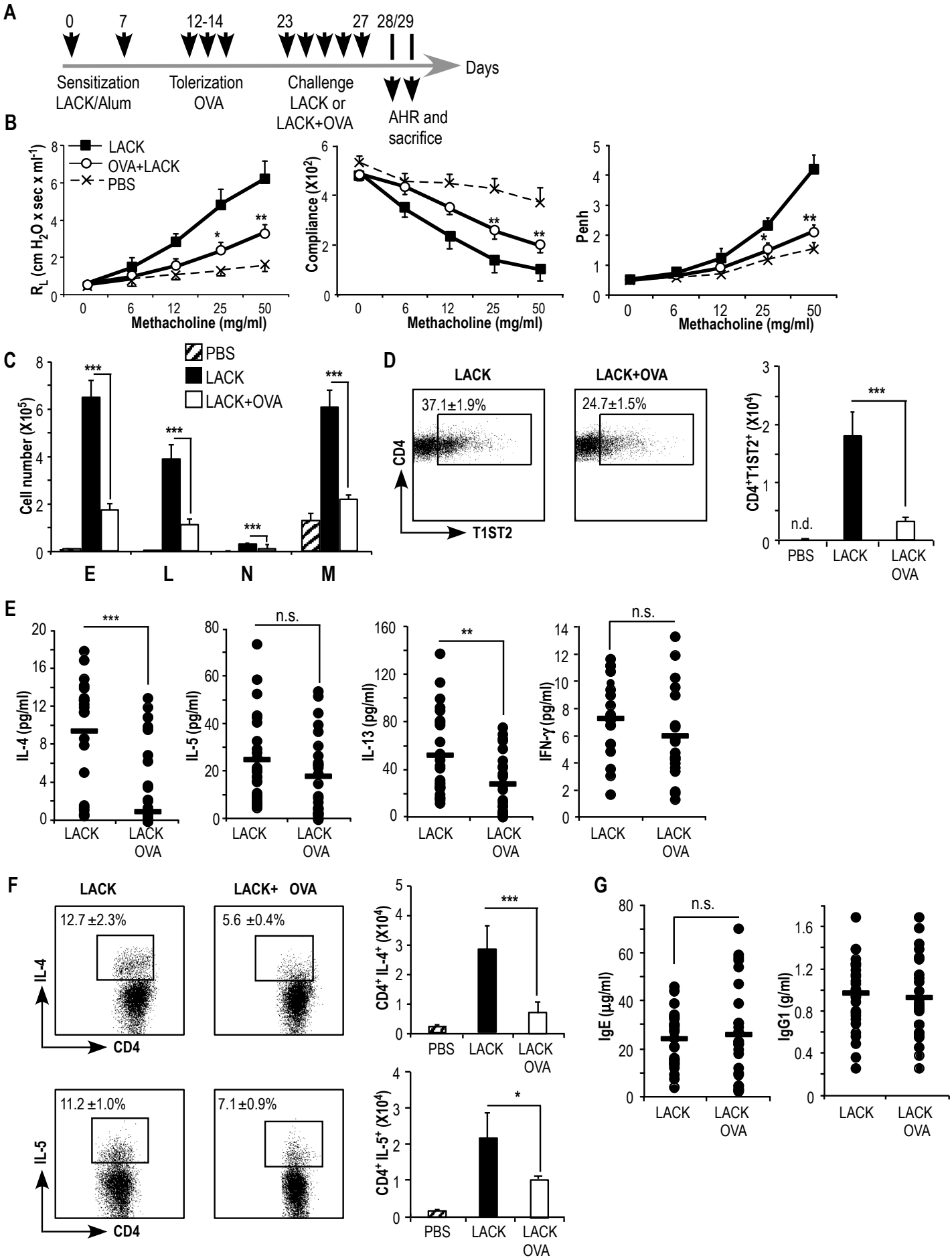

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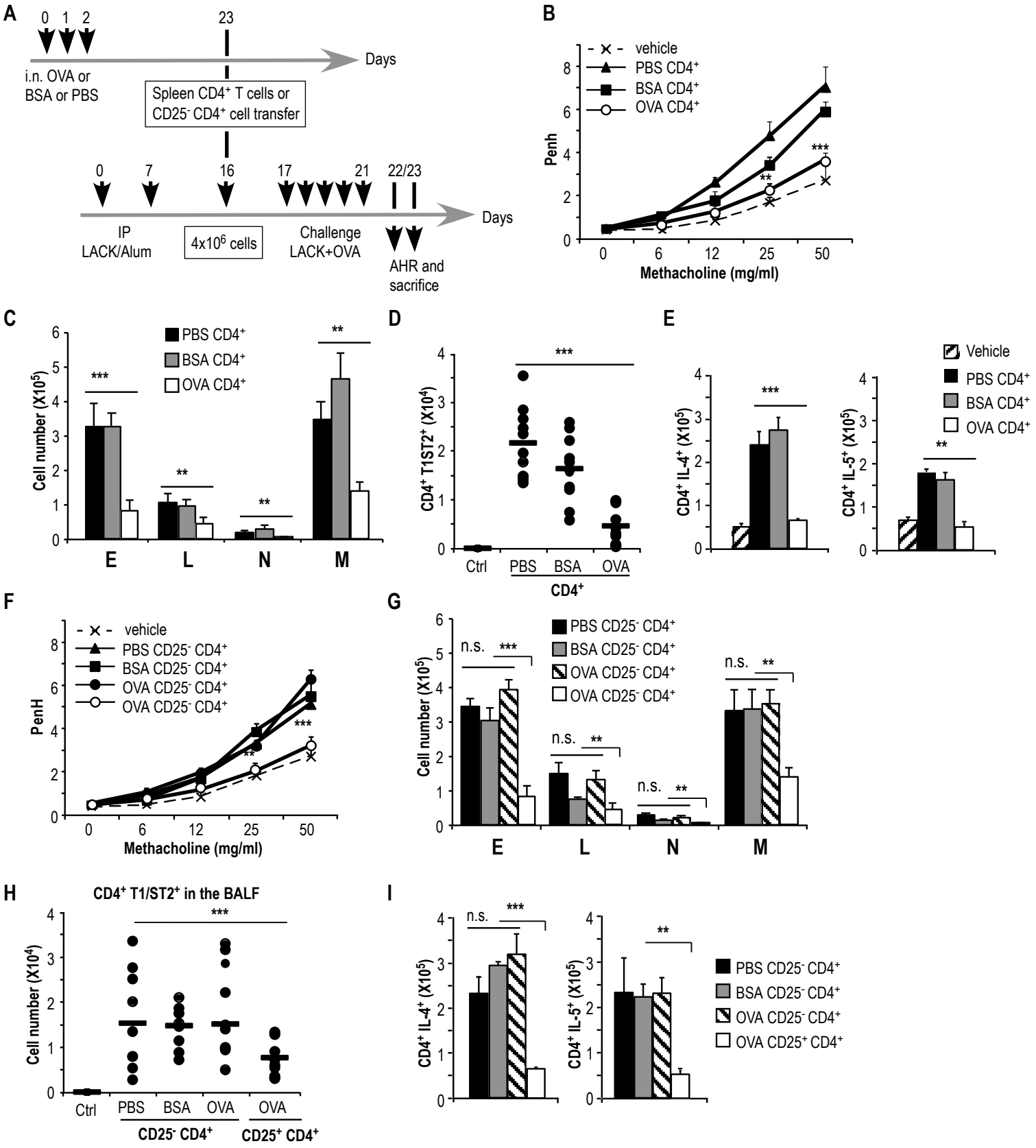

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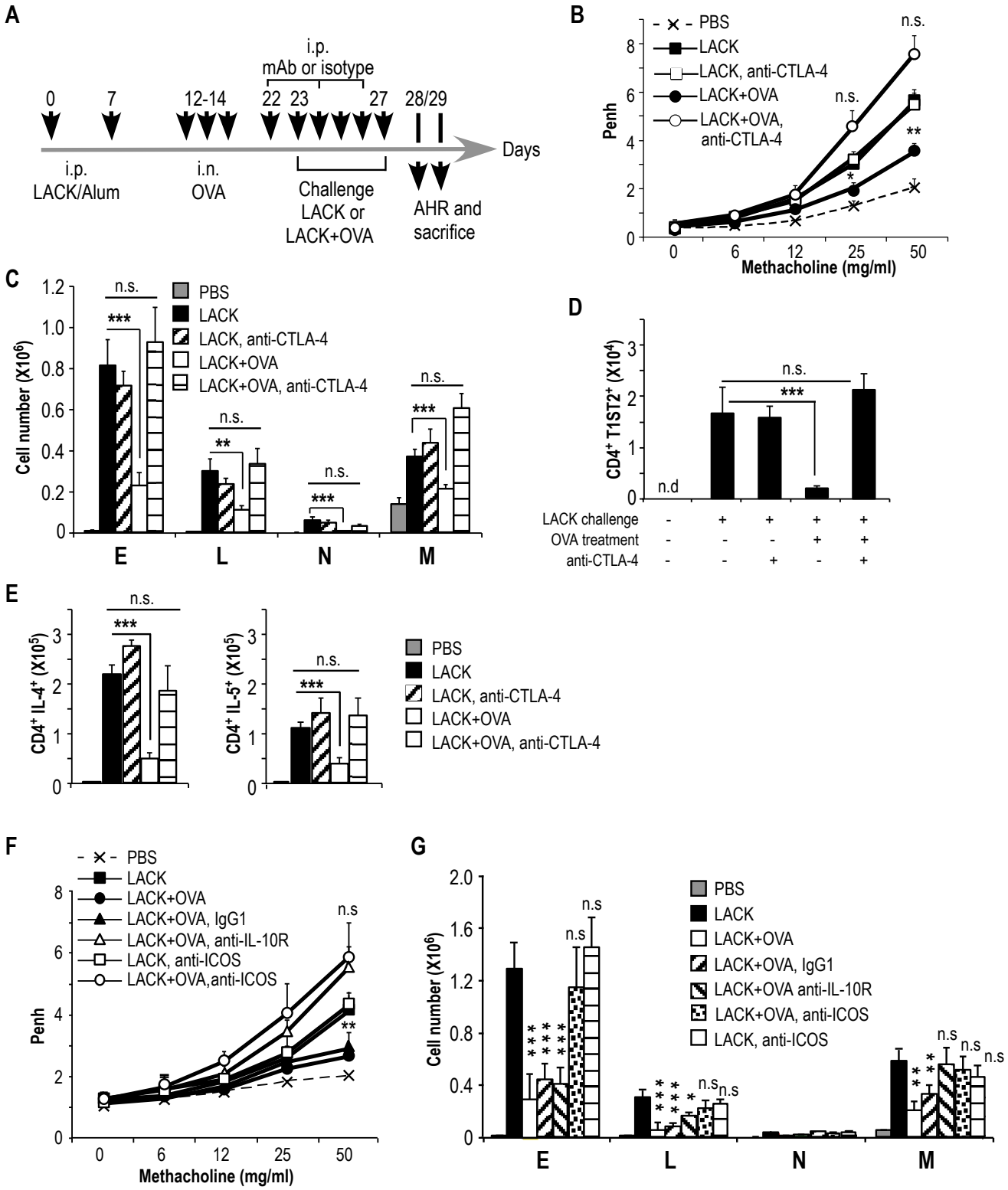

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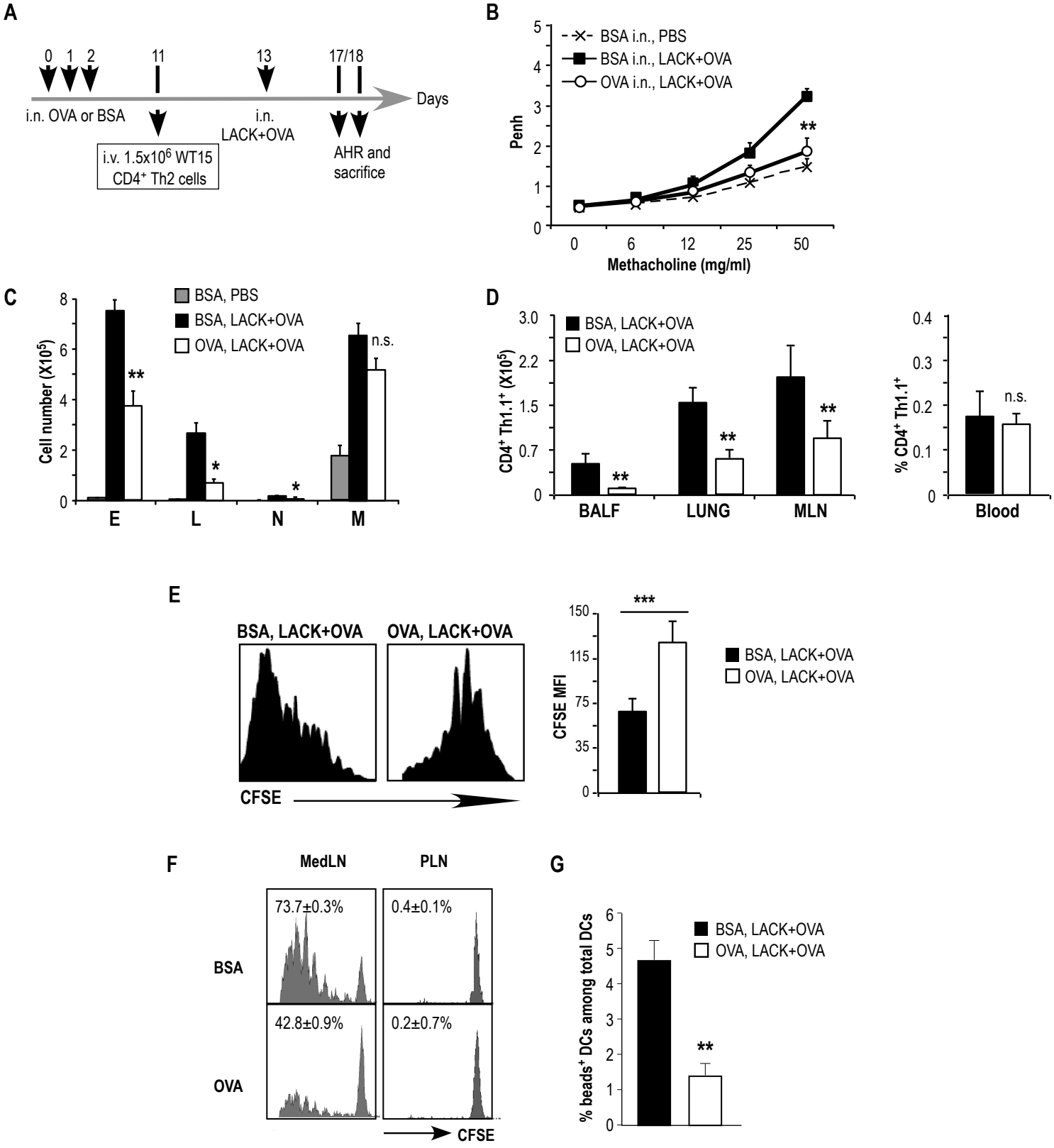

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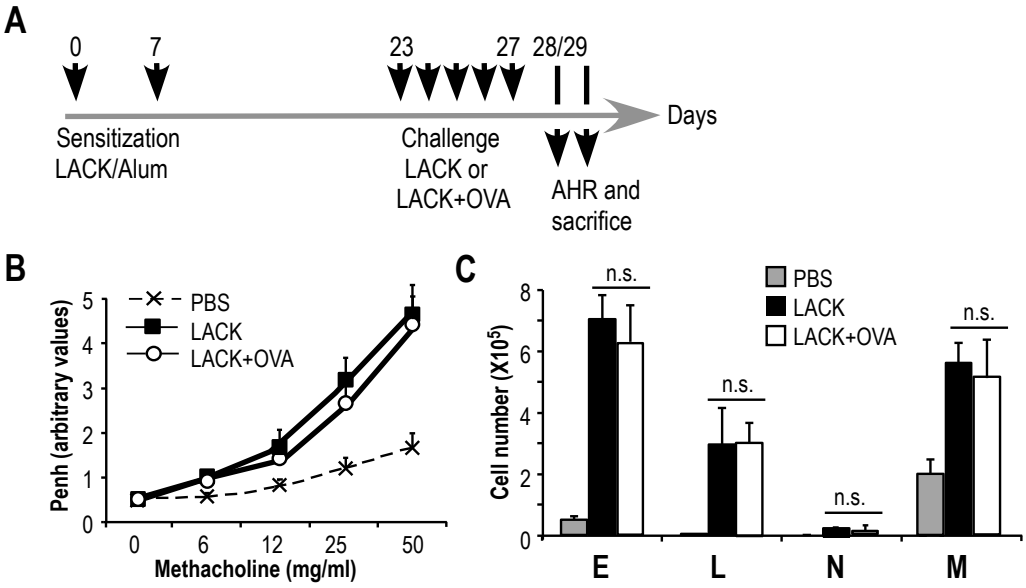

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